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CD56^{neg}CD16⁺ NK cells are activated mature NK cells with impaired effector function during HIV-1 infection

Jeffrey M Milush^{1*}, Sandra López-Vergès^{2,6}, Vanessa A York¹, Steven G Deeks³, Jeffrey N Martin⁴, Frederick M Hecht³, Lewis L Lanier² and Douglas F Nixon^{1,5}**Abstract**

Background: A subset of CD3^{neg}CD56^{neg}CD16⁺ Natural Killer (NK) cells is highly expanded during chronic HIV-1 infection. The role of this subset in HIV-1 pathogenesis remains unclear. The lack of NK cell lineage-specific markers has complicated the study of minor NK cell subpopulations.

Results: Using CD7 as an additional NK cell marker, we found that CD3^{neg}CD56^{neg}CD16⁺ cells are a heterogeneous population comprised of CD7⁺ NK cells and CD7^{neg} non-classical myeloid cells. CD7⁺CD56^{neg}CD16⁺ NK cells are significantly expanded in HIV-1 infection. CD7⁺CD56^{neg}CD16⁺ NK cells are mature and express KIRs, the C-type lectin-like receptors NKG2A and NKG2C, and natural cytotoxicity receptors similar to CD7⁺CD56⁺CD16⁺ NK cells. CD7⁺CD56^{neg} NK cells in healthy donors produced minimal IFN γ following K562 target cell or IL-12 plus IL-18 stimulation; however, they degranulated in response to K562 stimulation similar to CD7⁺CD56⁺ NK cells. HIV-1 infection resulted in reduced IFN γ secretion following K562 or cytokine stimulation by both NK cell subsets compared to healthy donors. Decreased granzyme B and perforin expression and increased expression of CD107a in the absence of stimulation, particularly in HIV-1-infected subjects, suggest that CD7⁺CD56^{neg}CD16⁺ NK cells may have recently engaged target cells. Furthermore, CD7⁺CD56^{neg}CD16⁺ NK cells have significantly increased expression of CD95, a marker of NK cell activation.

Conclusions: Taken together, CD7⁺CD56^{neg}CD16⁺ NK cells are activated, mature NK cells that may have recently engaged target cells.

Keywords: Natural killer cells, NK cells, CD7, Human immunodeficiency virus, HIV-1, HIV pathogenesis, CD56^{neg} NK cells

Background

Natural killer (NK) cells comprise 5–20% of peripheral blood mononuclear cells (PBMC) in humans and play a fundamental role in the defense against viral infections, as well as in tumor surveillance, and help shape adaptive immune responses through their production of cytokines [1–3]. NK cells are traditionally identified as CD3^{neg}, CD14^{neg}, CD19^{neg} lymphocytes expressing CD56 (neural cell adhesion molecule) and NKp46 [4], although not all human NK cells express NKp46 [1]. NK cells are

typically characterized into two main subsets; CD56^{dim}CD16⁺ NK cells, which comprise approximately 90% of circulating NK cells and are considered mature, and CD56^{bright}CD16^{neg/dim} NK cells, which represent approximately 10% and are considered immature [1,5]. A diverse array of activating and inhibitory receptors controls their function. Upon activation, NK cells secrete IFN γ and other cytokines and kill susceptible target cells [2].

Initial studies of NK cells during Human Immunodeficiency Virus (HIV-1) infection found significantly reduced absolute numbers of CD3^{neg}CD56⁺CD16⁺ NK cells with a concomitant increase in CD3^{neg}CD56^{neg}CD16⁺ cells [6–8]. In HIV-1-infected subjects, CD3^{neg}CD56^{neg}CD16⁺ cells are described as having decreased expression of activating

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receptors (i.e. NKp30 and NKp46) and increased expression of inhibitory receptors (i.e. LIR-1 and inhibitory KIR), and have poor cytolytic, proliferative, and cytokine-producing capabilities [8-14]. It has been hypothesized that the expansion of this defective CD3^{neg}CD56^{neg}CD16⁺ population might be one mechanism by which HIV-1 subverts the NK cell response.

Expansion of CD3^{neg}CD56^{neg}CD16⁺ cells has also been observed in other infectious diseases (i.e. hantavirus and chronic hepatitis C virus (HCV) infection) [15,16], as well as in ocular myasthenia gravis [17] and dermatomyositis [18]. Common to each of these pathological conditions is immune activation. Indeed, chronic immune activation is a hallmark of untreated HIV-1 disease and results in accelerated immunosenescence [19]. In healthy subjects, one study has suggested that NK cells might proliferate and die more rapidly than do T cells however this requires further investigation [20]. We recently demonstrated that CD57 is a marker of terminally differentiated NK cells [21], and that during acute cytomegalovirus (CMV) infection NKG2C^{hi}-expressing NK cells acquire expression of CD57 [22]. Importantly, treatment with antiviral drugs in HIV-1 and HCV infection or immunosuppressants for myasthenia gravis and dermatomyositis decrease the frequency of CD3^{neg}CD56^{neg}CD16⁺ cells over the course of months to levels found in healthy subjects [11,16-18].

There remain many unanswered questions regarding the phenotype, function, and origin of CD3^{neg}CD56^{neg}CD16⁺ cells in healthy individuals, and how they compare to the expanded population found during chronic infectious diseases such as HIV-1 infection. We recently demonstrated that including CD7 as an additional positive NK cell marker is an effective method for studying non-classical NK cell subsets [23]. Using CD7, we expand our collective knowledge about the phenotype and function of CD56^{neg}CD16⁺ cells in healthy and HIV-1-infected subjects. Based upon previous observations that chronic viremia is associated with an increased frequency of CD56^{neg}CD16⁺ cells, we tested the hypothesis that CD7 would refine the population of NK cells defined as CD56^{neg}CD16⁺ cells and provide evidence that CD7⁺CD56^{neg}CD16⁺ NK cells are mature NK cells.

Results

CD56^{neg}CD16⁺ cells are a mixed population of myeloid and NK cells that is expanded during chronic HIV-1 infection

PBMC from HIV-1-negative and HIV-1-infected subjects (Table 1) from the OPTIONS (early infection) and SCOPE (chronic infection) cohorts were gated on CD3^{neg}CD14^{neg}CD19^{neg} cells and the three major NK cell subsets were assessed: CD56^{bright}CD16^{neg}, CD56⁺CD16⁺, and CD56^{neg}CD16⁺ (Additional file 1). A significant decrease in the frequency of CD56^{bright}CD16^{neg} and CD56⁺CD16⁺ NK cells with a significant increase in

the frequency of CD56^{neg}CD16⁺ cells was observed in HIV-1-infected subjects, particularly during chronic HIV-1 infection (Figure 1A). These results are in agreement with previous studies describing CD56^{neg}CD16⁺ cells as highly expanded during chronic HIV-1 (and HCV) infections [8,12,16,24].

A recent gene expression study of CD56^{neg}CD16⁺ cells, traditional CD56⁺CD16⁺ NK cells, and myeloid cells found that the transcriptome of CD56^{neg}CD16⁺ cells was more similar to myeloid cells than to traditional CD56⁺CD16⁺ NK cells [25]. Therefore, we hypothesized that CD56^{neg}CD16⁺ cells are a mixed population of myeloid and NK cells that could be distinguished using CD7 [23]. In HIV-1-negative controls, less than 40% of CD56^{neg}CD16⁺ cells co-express CD7, indicating the CD56^{neg}CD16⁺ population is not a homogeneous population of NK cells (Figure 1B). During early HIV-1 infection, there is a significant expansion in the frequency of CD7⁺CD56^{neg}CD16⁺ NK cells that appears to persist into chronic HIV-1 infection; however, CD56^{neg}CD16⁺ cells remain a mixed myeloid and NK cell population (Figure 1B). In the absence of CD7 gating we did not observe the expansion of CD56^{neg}CD16⁺ cells during early infection (Figure 1A). Persistent HIV-1 viremia appears to be important for the accumulation of CD7⁺CD56^{neg}CD16⁺ NK cells [8,12,16,24]. No significant difference was observed in the frequency of CD7⁺CD56^{neg}CD16⁺ NK cells in healthy, non-HIV infected individuals, including both cytomegalovirus-seropositive and cytomegalovirus-seronegative subjects (Figure 1C). Taken together, CD56^{neg}CD16⁺ cells are a mixed population of cells, a subset of which are CD7⁺ NK cells that are expanded during persistent HIV-1 viremia.

CD7⁺CD56^{neg}CD16⁺ and CD7⁺CD56⁺CD16⁺ NK cells are phenotypically similar in healthy donors; however, HIV-1 infection significantly alters the phenotype of both NK cell subsets

In the absence of CD7 gating, CD56^{neg}CD16⁺ cells appear to have no or very low levels of NKp30 and granzyme B expression. However, when the CD7⁺CD56^{neg}CD16⁺ NK cells are gated and compared to mature CD7⁺CD56⁺CD16⁺ NK cells, little difference between the cell subsets was observed (Additional file 2). As these data are in disagreement with previous reports [12,26], we sought to further characterize CD7⁺CD56^{neg}CD16⁺ NK cells compared to CD7⁺CD56⁺CD16⁺ NK cells in healthy and HIV-1-infected individuals.

We assessed the expression of Killer cell Immunoglobulin-like Receptors (KIRs), C-type lectin-like receptors (NKG2A, NKG2C and CD161), and Natural Cytotoxicity Receptors (NCRs; NKp30, and NKp46), as well as receptors typically found on myeloid cells (CD33, CD13, and HLA-DR) in HIV-1-negative donors (Figure 2). No significant difference was observed in the frequency

Table 1 Early and chronic HIV-1-infected patient viral loads and CD4⁺ T cell counts

Patient ID	Cohort	Antiretroviral status	Viral load	CD4 ⁺ T cell count
647	OPTIONS	None	73988	594
683	OPTIONS	None	95903	480
722	OPTIONS	None	124070	522
730	OPTIONS	None	2185	594
792	OPTIONS	None	128274	281
804	OPTIONS	None	4812	800
830	OPTIONS	None	14207	850
858	OPTIONS	None	56133	466
876	OPTIONS	None	149012	343
1049	SCOPE	None	336630	218
1070	SCOPE	None	365000	331
1091	SCOPE	None	59935	288
1208	SCOPE	None	17641	1054
1217	SCOPE	None	19933	281
1311	SCOPE	None	56566	647
1335	SCOPE	None	50738	501
1538	SCOPE	None	47025	612
1566	SCOPE	None	34418	853
1571	SCOPE	None	21734	381
1587	SCOPE	None	13589	469
1588	SCOPE	None	25552	891
1596	SCOPE	None	23364	621
1597	SCOPE	None	12593	498
1606	SCOPE	None	24458	678
1654	SCOPE	None	91597	441
1662	SCOPE	None	236000	584
1679	SCOPE	None	145465	288
4015	SCOPE	None	63400	714

of KIR2DL1- and KIR2DL3-bearing cells between CD7⁺CD56⁺CD16⁺ and CD7⁺CD56^{neg}CD16⁺ NK cells, although CD7⁺CD56^{neg}CD16⁺ NK cells had a significantly lower frequency of KIR3DS1/DL1-positive NK cells. The density of KIR2DL1 and KIR2DL3 as measured by mean fluorescent intensity (MFI) was significantly lower on CD7⁺CD56^{neg}CD16⁺ NK cells, whereas no significant difference in MFI of KIR3DS1/DL1 was observed between the NK cell subsets (Figure 2A-C). With regard to the C-type lectin-like receptors, similar frequencies of both NK cell subsets expressed NKG2A and NKG2C. While both NK cell subsets had similar densities of NKG2C expression, the density of NKG2A was significantly lower on CD7⁺CD56^{neg}CD16⁺ NK cells (Figure 2D-E). No significant differences were observed in the frequency of NKp30 and NKp46 between the NK cell subsets (Figure 2G-H). In contrast to previous studies, NKp30 and NKp46 were expressed at significantly greater

densities on CD7⁺CD56^{neg}CD16⁺ NK cells compared with CD7⁺CD56⁺CD16⁺ NK cells (Figure 2G-H). By including all CD7⁺CD56⁺ NK cells (i.e. CD56^{br}CD16^{neg}, CD56^{br}CD16^{dim}, and CD56⁺CD16⁺) in the analyses as was done by Mavilio *et al.* [12], the MFI of NKp30 and NKp46 expression remained significantly greater in CD7⁺CD56^{neg}CD16⁺ NK cells compared with total CD7⁺CD56⁺ NK cells and CD7⁺CD56⁺CD16⁺ NK cells (Additional file 3). However, total CD7⁺CD56⁺ NK cells showed a trend ($p = 0.070$) toward greater NKp46 density compared to CD7⁺CD56⁺CD16⁺ NK cells (Additional file 3B). No significant differences were observed in the frequency or density of CD161 expression between CD7⁺CD56⁺CD16⁺ and CD7⁺CD56^{neg}CD16⁺ NK cells (Figure 2F). A prior study of CD56^{neg}CD16⁺ NK cells indicated a significantly greater expression of the inhibitory receptor LIR-1 on CD56^{neg}CD16⁺ compared to CD56⁺CD16⁺ NK cells [12]. However, using

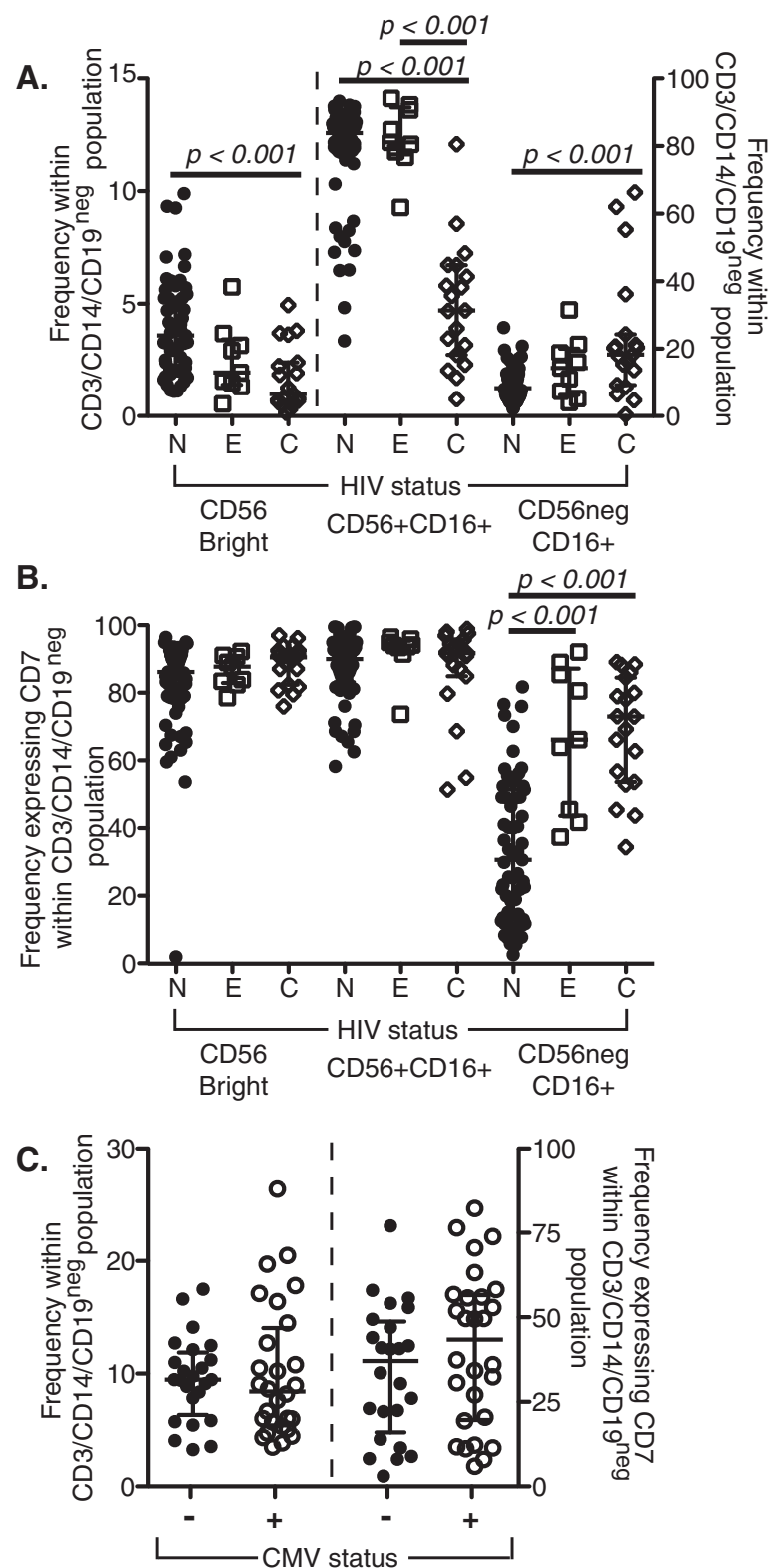


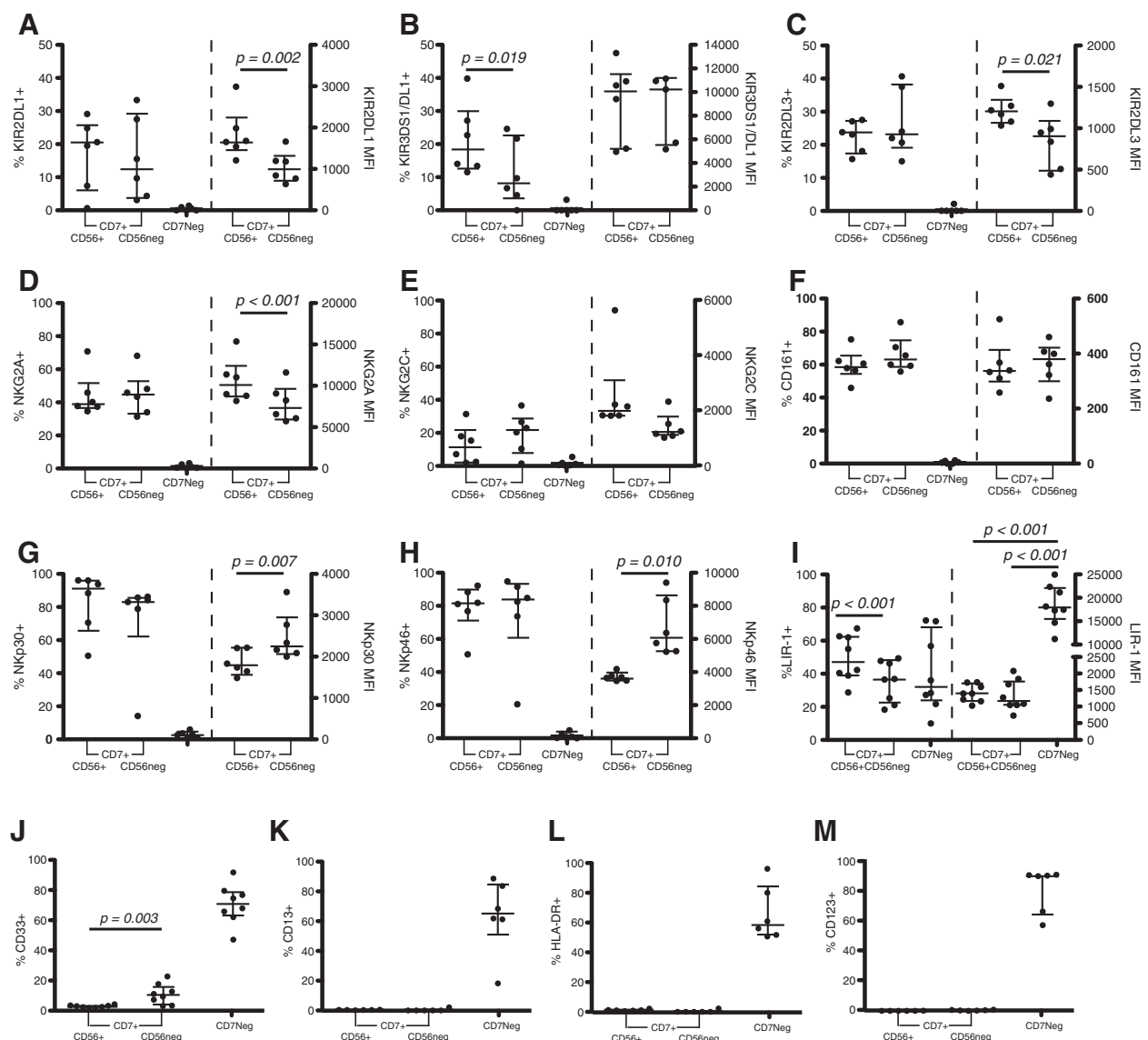
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Figure 1 Distribution of NK cell subsets. **A**, Frequency of classically defined NK cell subsets within the $CD3^{neg}CD14^{neg}CD19^{neg}$ population of healthy donors (\square , $n = 63$) and early- (\square , $n = 9$) and chronically- (\blacklozenge , $n = 19$) HIV-1-infected subjects. **B**, Frequency of $CD3^{neg}CD14^{neg}CD19^{neg}$ cells expressing CD7 within the $CD56^{bright}CD16^{neg}$, $CD56^{+}CD16^{+}$ and $CD56^{neg}CD16^{+}$ subsets in healthy (\square , $n = 63$) and early- (\square , $n = 9$) and chronically- (\blacklozenge , $n = 19$) HIV-1-infected subjects. **C**, Frequency of $CD56^{neg}CD16^{+}$ cells within the $CD3^{neg}CD14^{neg}CD19^{neg}$ population (left axis) and of $CD3^{neg}CD14^{neg}CD19^{neg}$ cells expressing CD7 within the $CD56^{neg}CD16^{+}$ subset (right axis) of CMV-negative (closed circles) and CMV-positive (open circles) healthy donors. The median and 25th and 75th percentile are indicated on each graph.

CD7 to further delineate NK cells, we observed that LIR-1 density on $CD7^{+}CD56^{+}CD16^{+}$ and $CD7^{+}CD56^{neg}CD16^{+}$ NK cells was not significantly different (Figure 2I). Indeed, the apparent higher levels of LIR-1 expression on

$CD56^{neg}CD16^{+}$ cells was likely due to the contamination with $CD7^{neg}CD56^{neg}CD16^{+}$ myeloid cells that have five-fold greater LIR-1 density than $CD7^{+}$ NK cells. Importantly, $CD7^{neg}CD56^{neg}CD16^{+}$ cells did not express any of



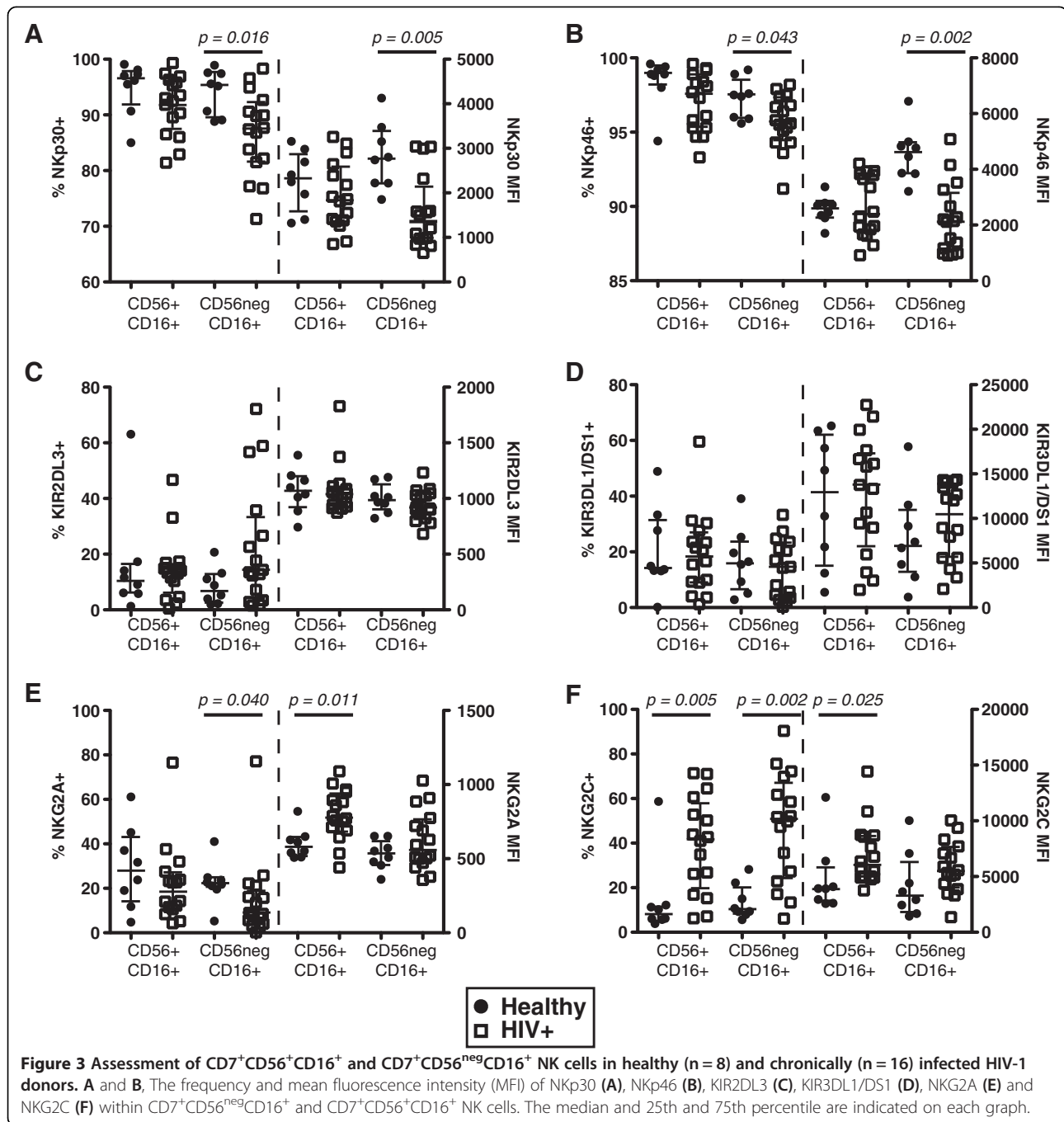
these NK cell-associated receptors. However, high frequencies of CD7^{neg}CD56^{neg}CD16⁺ cells did express CD33, CD13, and HLA-DR, markers that are classically expressed by myeloid cells (Figure 2J-L). Recently, Bigley *et al.* observed that a subset of CD123⁺ plasmacytoid dendritic cells express CD7 [27]. Although approximately 80% of the CD7^{neg}CD56^{neg}CD16⁺ cells expressed CD123, no expression of CD123 was observed on CD7⁺CD56⁺CD16⁺ or CD7⁺CD56^{neg}CD16⁺ NK cells (Figure 2M). These results are in agreement with Bigley *et al.* who reported that CD7⁺ CD123⁺ plasmacytoid dendritic cells were CD16-negative [27]. The phenotypic profile of CD7^{neg}CD56^{neg}CD16⁺ cells is largely consistent with non-classical CD14^{neg}CD16⁺ monocytes and a subset of dendritic cells (DCs) designated slanDCs [28] that fall within the lymphoid gate based on light scattering properties.

To determine the impact of HIV-1 infection on the phenotype of CD7⁺CD56⁺CD16⁺ and CD7⁺CD56^{neg}CD16⁺ NK cells, KIR, C-type lectin-like receptors, and NCRs were assessed in 16 chronically HIV-1-infected subjects and 8 healthy controls (Figure 3). HIV-1-infected subjects trended towards a reduced frequency of NKp30- and NKp46-positive CD7⁺CD56⁺CD16⁺ NK cells compared to healthy controls. However, the frequency of NKp30- and NKp46-expressing CD7⁺CD56^{neg}CD16⁺ NK cells and the density of NKp30 and NKp46 expression on CD7⁺CD56^{neg}CD16⁺ NK cells were significantly lower in HIV-1-infected subjects. Importantly, there were no significant differences in the frequencies of cells expressing NCRs or the density of the NCRs between CD7⁺CD56⁺CD16⁺ and CD7⁺CD56^{neg}CD16⁺ NK cells in the HIV-1-infected subjects (Figure 3A and B). Assessment of KIR2DL3 and KIR3DL1/DS1 did not reveal any significant differences between subsets of NK cells or the HIV-1 infection status of the subject (Figure 3C and D). A significantly lower frequency of CD7⁺CD56^{neg}CD16⁺ NK cells within HIV-1-infected subjects expressed NKG2A, while the density of NKG2A expression was significantly greater on CD7⁺CD56⁺CD16⁺ NK cells in HIV-1-infected subjects (Figure 3E). The frequency of NK cells and density of the activating receptor NKG2C is elevated in CMV-infected individuals [22,29,30], a co-infection that is highly prevalent (>98% in the SCOPE cohort [31]) in HIV-1-infected subjects. To this end, it was not unexpected to observe that the frequency of NKG2C-bearing cells was significantly greater on both subsets of NK cells in HIV-1-infected subjects compared to healthy controls (Figure 3F). Furthermore, the density of NKG2C expression was significantly greater on CD7⁺CD56⁺CD16⁺ NK cells in HIV-1-infected compared to uninfected individuals. However, no significant differences were observed between CD7⁺CD56⁺CD16⁺ and CD7⁺CD56^{neg}CD16⁺ NK cell subsets within the HIV-1-infected subjects (Figure 3F). The increased frequency and density of NKG2C⁺ NK cells in HIV-1-infected subjects likely represents the higher

prevalence of cytomegalovirus infection or reactivation in these subjects [31]. Taken together these results indicate that HIV-1 infection has a significant impact on the overall NK cell phenotype. However, with the exception of NKp30 and NKp46, CD7⁺CD56^{neg}CD16⁺ NK cells do not appear to be significantly altered compared to CD7⁺CD56⁺CD16⁺ NK cells within HIV-1-infected subjects.

CD7⁺CD56^{neg}CD16⁺ NK cells are mature NK cells

To determine whether differences in maturation existed between CD7⁺CD56^{neg}CD16⁺ and CD7⁺CD56⁺CD16⁺ NK cells, we assessed the expression of CD16, CD57, Siglec-7, CD62L, and CXCR3. A significantly greater frequency of CD7⁺CD56⁺CD16⁺ NK cells in HIV-1-infected subjects expressed CD57 compared to healthy subjects. Although CD57 expression on CD7⁺CD56⁺CD16⁺ and CD7⁺CD56^{neg}CD16⁺ NK cells in healthy controls was not significantly different, the frequency of CD57⁺CD7⁺CD56^{neg}CD16⁺ NK cells in HIV-1-infected subjects was significantly less compared to CD57⁺CD7⁺CD56⁺CD16⁺ NK cells (~44% versus ~54% respectively; Figure 4A). When we separated healthy subjects by CMV serology status, we did not observe a significant difference in CD57 expression between the NK cell subsets (data not shown). CD16 expression increases with NK cell maturation [21]. We observed that early HIV-1 infection resulted in a significant increase in CD16 expression on both subsets of NK cells compared to healthy controls (Figure 4B left half). Because the studies were performed at different times, we were unable to directly compare the density of CD16 expression (based on MFI) in early and chronic HIV-1 infection. However, in contrast to early infection, CD16 expression did not appear to differ significantly on CD7⁺CD56⁺CD16⁺ or CD7⁺CD56^{neg}CD16⁺ NK cell subsets in chronically HIV-1-infected subjects compared to healthy controls (Figure 4B, right half). Recently, HIV-1 infection was reported to induce a rapid and sustained decrease in Siglec-7 expression on NK cells, which is associated with impaired function [11]. We observed that greater than 90% of CD7⁺CD56⁺CD16⁺ NK cells in healthy donors expressed Siglec-7, whereas approximately 50% of this NK cell subset expressed Siglec-7 in HIV-1-infected subjects (Figure 4C). Furthermore, we found that a lower frequency of CD7⁺CD56^{neg}CD16⁺ NK cells expressed Siglec-7 in both healthy controls and HIV-1-infected subjects, but the loss of Siglec-7 on CD7⁺CD56^{neg}CD16⁺ NK cells of HIV-1-infected subjects was significantly greater (Figure 4C). During NK cell maturation from an immature CD56^{bright}CD16^{neg} to a mature CD56⁺CD16⁺ phenotype, CD62L and CXCR3 expression are decreased [32-34]. In accordance with a mature phenotype, CD7⁺CD56^{neg}CD16⁺ NK cells have similar CD62L expression compared to CD7⁺CD56⁺CD16⁺ NK



cells, which is significantly lower than that expressed by CD7⁺CD56^{bright}CD16^{neg} immature NK cells (Figure 4D). Similarly, less than 5% of both CD7⁺CD56^{neg}CD16⁺ and CD7⁺CD56⁺CD16⁺ NK cells express CXCR3 (Figure 4E). By comparison, approximately 70% of CD7⁺CD56^{bright}CD16^{neg} immature NK cells express CXCR3, and HIV-1 infection is associated with a significantly lower frequency of CXCR3⁺ CD7⁺CD56^{bright}CD16^{neg} immature NK cells compared to healthy controls (Figure 4E). In healthy controls, CXCR3 is

expressed by a significantly lower frequency of CD7⁺CD56^{neg}CD16⁺ compared to CD7⁺CD56⁺CD16⁺ NK cells (Figure 4E). HIV-1 infection was associated with a significant decrease in the frequency of CXCR3⁺CD7⁺CD56⁺CD16⁺ NK cells (Figure 4E). Taken together, CD7⁺CD56^{neg}CD16⁺ and CD7⁺CD56⁺CD16⁺ NK cells are mature NK cell subsets with at least a fraction being terminally differentiated CD57⁺ NK cells; however, HIV-1-infection significantly alters the differentiation of both NK cell subsets.

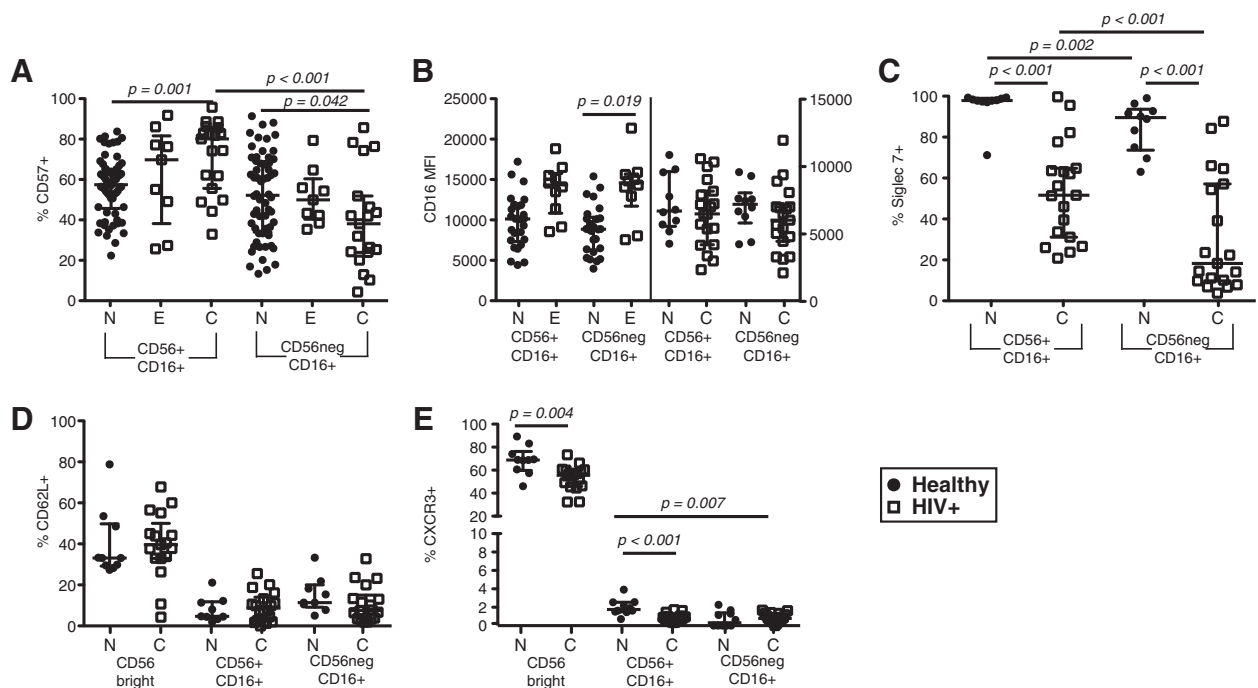


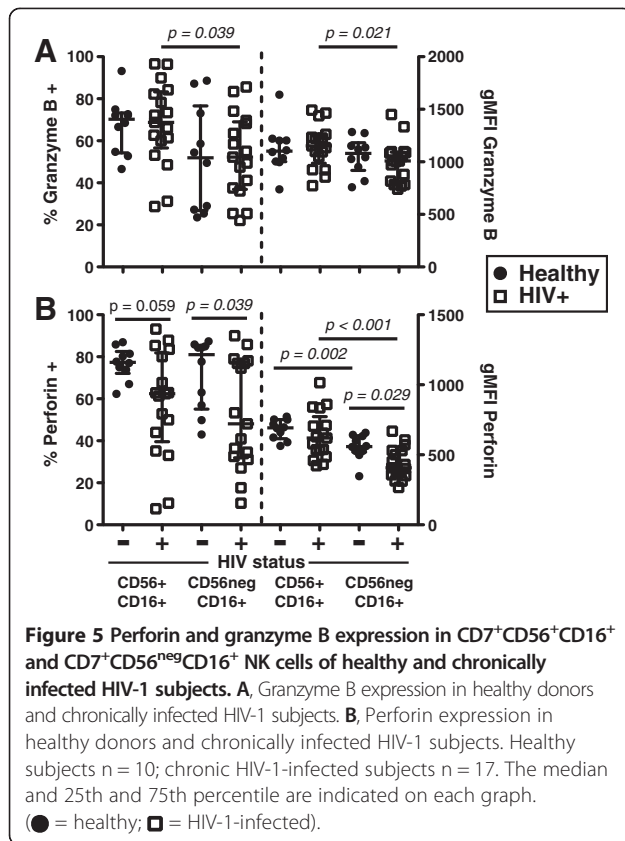
Figure 4 Assessment of maturation in NK cell subsets of healthy and HIV-1-infected subjects. **A**, Frequency of $CD7^+CD56^+CD16^+$ and $CD7^+CD56^{neg}CD16^+$ NK cells expressing CD57 in healthy ($n = 62$) and early ($n = 9$) and chronically ($n = 19$) HIV-1-infected subjects. **B**, The MFI of CD16 expression was assessed on $CD7^+CD56^+CD16^+$ and $CD7^+CD56^{neg}CD16^+$ NK cells in healthy ($n = 52$) and early ($n = 9$) HIV-1-infected subjects (left half), and in healthy ($n = 10$) and chronically HIV-1-infected subjects ($n = 19$) (right half). The healthy controls and early HIV-1 subjects were stained and analyzed in a different study with an anti-CD16 antibody conjugated to a different fluorophore than the healthy controls and chronic HIV-1-infected subjects. Therefore, early and chronic HIV-1-infected subjects cannot be directly compared with regard to mean fluorescent intensity. **C**, The frequency of $CD7^+CD56^+CD16^+$ and $CD7^+CD56^{neg}CD16^+$ NK cells expressing Siglec-7. **D-E**, The frequency of $CD7^+CD56^{bright}CD16^{neg}$, $CD7^+CD56^+CD16^+$ and $CD7^+CD56^{neg}CD16^+$ NK cells expressing **D**) CD62L and **E**) CXCR3. The same healthy ($n = 10$) and chronic HIV-1-infected subjects ($n = 19$) used in 4B were used to assess Siglec-7, CD62L and CXCR3. The median and 25th and 75th percentile are indicated on each graph. (● = healthy; □ = HIV-1-infected).

HIV-1 infection has a significant impact on the function of both NK cell subsets

Granzyme B and perforin are important effector molecules expressed by mature NK cells. In healthy controls, no statistically significant differences in the frequency of NK cells expressing granzyme B or the amount of granzyme B in the NK cell subsets was observed, although a trend toward a decreased frequency of $CD7^+CD56^{neg}CD16^+$ NK cells expressing granzyme B was observed (Figure 5A). In contrast, the frequency of granzyme B-positive NK cells and the amount of granzyme B expression were significantly lower in $CD7^+CD56^{neg}CD16^+$ NK cells of HIV-1-chronically-infected subjects. No statistically significant differences were observed between healthy control and HIV-1-chronically-infected subjects in granzyme B expression within the $CD7^+CD56^+CD16^+$ or $CD7^+CD56^{neg}CD16^+$ NK cell subsets (Figure 5A). A significantly lower amount of perforin was observed in $CD7^+CD56^{neg}CD16^+$ NK cells of both healthy controls and HIV-1-chronically-infected subjects compared with $CD7^+CD56^+CD16^+$ NK cells (Figure 5B). The frequency of cells expressing perforin

in both $CD7^+CD56^+CD16^+$ and $CD7^+CD56^{neg}CD16^+$ NK cell populations was significantly lower in HIV-1-chronically-infected subjects. Furthermore, the amount of perforin in $CD7^+CD56^{neg}CD16^+$ NK cells was significantly lower compared to $CD7^+CD56^+CD16^+$ NK cells in HIV-1-chronically-infected subjects (Figure 5B). Taken together, significantly reduced granzyme B and perforin expression in $CD7^+CD56^{neg}CD16^+$ NK cells of HIV-1-chronically-infected subjects might indicate this NK cell subset has recently degranulated in response to an encounter with target cells.

$CD56^{neg}CD16^+$ NK cells have been described as anergic and having poor cytokine and cytotoxic function *in vitro* [9,10,12]. Indeed, in both healthy controls and HIV-1-infected subjects, $CD7^+CD56^{neg}CD16^+$ NK cells expressed significantly less IFN γ compared to $CD7^+CD56^+CD16^+$ NK cells following K562 target cell stimulation (Figure 6A-B). IL-12 plus IL-18 stimulation did induce IFN γ secretion from $CD7^+CD56^{neg}CD16^+$ NK cells in healthy controls, albeit less than $CD7^+CD56^+CD16^+$ NK cells (Figure 6A). $CD7^+CD56^+CD16^+$ NK cells from HIV-1-chronically-



infected subjects had a significantly lower frequency of IFN γ -secreting cells in response to either K562 target cells ($p = 0.033$) or IL-12 plus IL-18 stimulation ($p = 0.011$) (Figure 6A-B). CD7⁺CD56^{neg}CD16⁺ NK cells from HIV-1-chronically-infected subjects had a significantly higher frequency of IFN γ ⁺ cells ($p = 0.013$) compared to healthy controls in the unstimulated condition. IFN γ responses induced by K562 target cells in CD7⁺CD56^{neg}CD16⁺ NK cells from healthy and HIV-1-chronically-infected subjects were negligible. CD7⁺CD56^{neg}CD16⁺ NK cells from HIV-1-chronically-infected subjects failed to produce IFN γ following cytokine stimulation (Figure 6B). In contrast to IFN γ responses, no significant differences in the frequencies of CD107a-expressing CD7⁺CD56⁺CD16⁺ and CD7⁺CD56^{neg}CD16⁺ NK cells were observed following K562 target stimulation in healthy controls. Indeed, both subsets of NK cells were capable of degranulating in response to target cell stimulation (Figure 6C). In HIV-1-chronically-infected subjects, CD7⁺CD56^{neg}CD16⁺ NK cells had a significantly higher frequency of CD107a expression in the unstimulated condition (Figure 6D). In contrast to their CD7⁺CD56⁺CD16⁺ NK cell counterpart, CD7⁺CD56^{neg}CD16⁺ NK cells from HIV-1-chronically-infected subjects did not significantly degranulate following K562 stimulation (Figure 6C-D). A comparison of CD107a⁺ cells in CD7⁺CD56⁺CD16⁺ and CD7⁺CD56^{neg}CD16⁺ NK

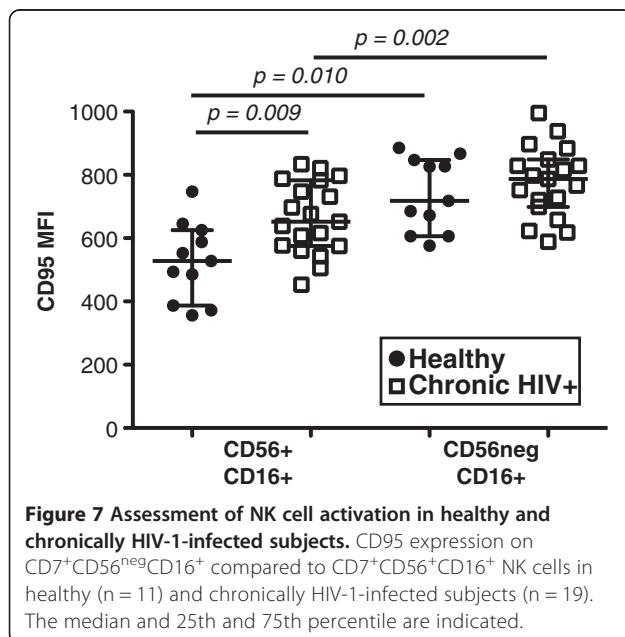
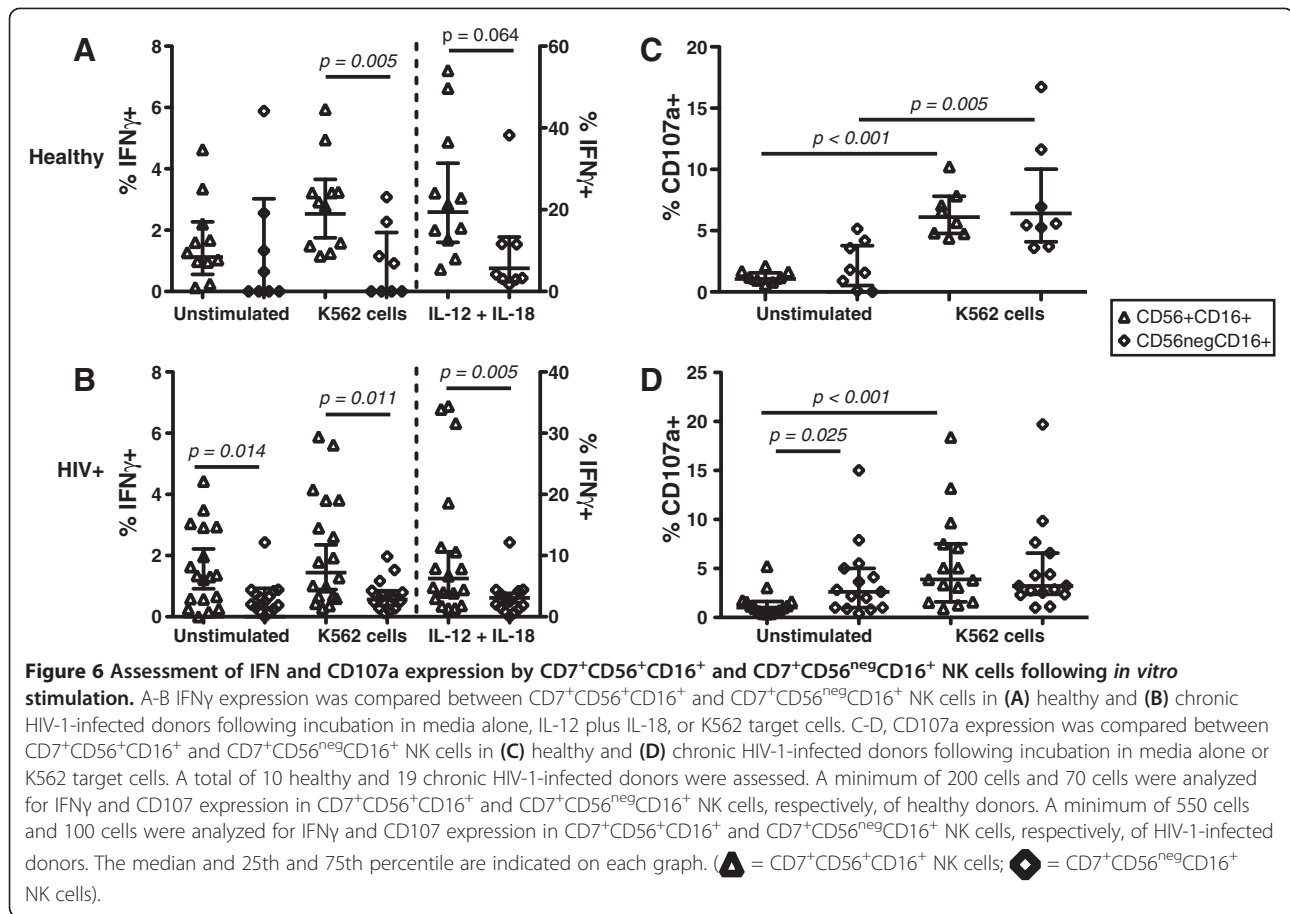
cells in healthy and HIV-1-chronically-infected subjects did not result in any statistically significant differences. Taken together, CD7⁺CD56^{neg}CD16⁺ NK cells from healthy controls were capable of degranulating following target cell stimulation. However, HIV-1 infection resulted in a significant defect in IFN γ secretion in both NK cell subsets and in degranulation in CD7⁺CD56^{neg}CD16⁺ NK cells compared to CD7⁺CD56⁺CD16⁺ NK cells. Interestingly, HIV-1 infection also resulted in a higher frequency of IFN γ secretion and degranulation by CD7⁺CD56^{neg}CD16⁺ NK cells in unstimulated conditions.

Increased CD95 expression by CD7⁺CD56^{neg}CD16⁺ NK cells

Granzyme B and perforin expression are lower in unstimulated CD7⁺CD56^{neg}CD16⁺ NK cells compared to CD7⁺CD56⁺CD16⁺ NK cells (Figure 5). Furthermore, unstimulated CD7⁺CD56^{neg}CD16⁺ NK cells have significantly higher basal CD107a expression compared to CD7⁺CD56⁺CD16⁺ NK cells, particularly in HIV-1-infected subjects (Figure 6). These data suggest that CD7⁺CD56^{neg}CD16⁺ NK cells might represent an activated subset of NK cells that have recently engaged a target. Previous studies have shown that resting NK cells express a basal level of the apoptosis-inducing receptor CD95 on their cell surface and that upon activation either through Fc receptor [35] or cytokine stimulation [36], CD95 is significantly upregulated on NK cells. Kottlil *et al.* recently demonstrated that CD95 expression is increased on NK cells in HIV-1-infected subjects predominantly within the CD56⁺CD16⁺ NK cell subset [37]. Indeed, we observed that CD95 is significantly increased on both subsets of NK cells in HIV-1-chronically-infected subjects compared to healthy controls, but is particularly increased on CD7⁺CD56^{neg}CD16⁺ NK cells in both healthy controls and HIV-1-chronically-infected subjects (Figure 7). These data suggest NK cells are activated during HIV-1 infection and that CD7⁺CD56^{neg}CD16⁺ NK cells might represent NK cells that have recently engaged a target cell.

Discussion

The lineage and biological role of CD3^{neg}CD56^{neg}CD16⁺ cells has remained poorly understood. The lack of a truly NK cell-specific marker and changes in cell phenotype during HIV-1 infection, as well as the potential overlap of phenotypic and functional properties of NK cells with other immune cell subsets, creates difficulties in studying rare and non-classical NK cell subsets. In humans, NK cells are most commonly identified as CD3^{neg}CD56⁺ lymphocytes with functionally distinct subsets being further defined by CD16 expression. However, CD16 is expressed on many other immune cells, including granulocytes and a subset of dendritic cells (DCs), designated



slanDCs [28]. Importantly, slanDCs do not express CD56; however, they do express CD13, CD33, HLA-DR, and high surface expression of CD16, which is the phenotype we observed on the CD7^{neg}CD56^{neg} population of cells. Therefore, caution is needed in interpreting the frequencies and functions of CD56^{neg} NK cells. Here, we built upon our previous work using CD7 as an informative marker of NK cells and demonstrated that CD3^{neg}CD56^{neg}CD16⁺ cells are a mixed population of CD7⁺ NK cells and CD7^{neg} myeloid cells present in both healthy and HIV-1-infected subjects. Previously published microarray analyses support this observation as the CD3^{neg}CD56^{neg}CD16⁺ population exhibited a transcriptome more closely related to myeloid cells than traditional CD3^{neg}CD56⁺CD16⁺ NK cells [25]. Our study has provided a more detailed understanding of the phenotype, function, and possible origin during chronic HIV-1 infection, of CD7⁺CD56^{neg}CD16⁺ NK cells in healthy and HIV-1-infected subjects.

Little is known regarding the phenotype and function of CD56^{neg}CD16⁺ cells in healthy subjects and whether these cells are similar to those observed in chronic viral infections. In HIV-1-negative subjects, we observed minor alterations in KIR, NCR, or C-type lectin-like receptors between the two NK cell subsets. In contrast to a previous

report indicating a reduced density of NKp30 on CD56^{neg}CD16⁺ cells in healthy donors [38], we observed that CD7⁺CD56^{neg}CD16⁺ NK cells actually had increased NKp30 expression. Functionally, CD7⁺CD56^{neg}CD16⁺ NK cells had impaired cytokine responses following K562 target cell or IL-12 plus IL-18 stimulation; however, they degranulated (as measured by CD107a-expression) similar to CD7⁺CD56⁺CD16⁺ NK cells following K562 target cell stimulation. By removing CD7^{neg} non-NK cells, we demonstrated that CD7⁺CD56^{neg}CD16⁺ NK cells do not have as significantly altered of a phenotype as previously shown. While our results confirm that CD56^{neg} NK cells do exist in healthy donors, using CD7 provided for a precise determination of the frequency of CD56^{neg} NK cells present in the blood of healthy donors and allowed for analyses of their phenotype and functional characteristics.

Numerous studies have found decreases in both frequency and absolute numbers of NK cells defined as CD3^{neg}CD56⁺ with a concomitant increase in a population of CD3^{neg}CD56^{neg}CD16⁺ cells in HIV-1 infection [6-8]. Our results confirm the expansion of a CD56^{neg} NK cell population; however, this population is not homogenous (<70% were CD7⁺CD56^{neg}CD16⁺ NK cells). KIR expression was not significantly altered on either CD7⁺CD56⁺ or CD7⁺CD56^{neg} NK cells during HIV-1 infection compared to healthy donors. While HIV-1 infection was not associated with significant alterations in the frequency of cells expressing NKp30 or NKp46 in the CD7⁺CD56⁺ NK cell population, we did observe significant reductions in density of these receptors on CD7⁺CD56^{neg} NK cells. During HIV-1 infection, CD7⁺CD56^{neg} NK cells appeared to be mature; however, there were fewer CD57⁺ terminally differentiated CD7⁺CD56^{neg} compared to CD7⁺CD56⁺ NK cells. We recently demonstrated that CD57 is a marker of terminally differentiated NK cells [21], and that CD57⁺NKG2C^{hi} NK cells take weeks to develop *in vivo* following cytomegalovirus infection [22]. One possible explanation for the expansion of this CD7⁺CD56^{neg} NK cell subset is that during HIV-1 infection, the already rapid turnover of NK cells [20] is further accelerated, preventing this subset of NK cells from terminally differentiating and gaining CD57 expression. Furthermore, our observation that CD7⁺CD56^{neg} NK cells express more of the apoptosis-inducing receptor CD95 further suggests these cells might undergo apoptosis prior to becoming terminally differentiated.

An alternative hypothesis is that the expanded population of CD7⁺CD56^{neg}CD16⁺ NK cells during HIV-1 infection represents mature NK cells that arise at least in part when CD7⁺CD56⁺CD16⁺ NK cells engage target cells. NK cells are capable of killing multiple target cells and this results in a reduced, but never complete, loss of perforin and granzyme B [39]. Here, decreased granzyme B and perforin expression and increased expression of CD107a in the absence

of *ex vivo* stimulation within the CD7⁺CD56^{neg}CD16⁺ NK cells, particularly of HIV-1-infected subjects, suggests that CD7⁺CD56^{neg}CD16⁺ NK cells may have recently engaged target cells *in vivo*. This is further supported by the increased expression of CD95 on CD7⁺CD56^{neg}CD16⁺ NK cells, indicating these cells are more activated than their CD7⁺CD56⁺CD16⁺ NK cell counterparts.

Importantly, the frequency of CD7⁺CD56^{neg}CD16⁺ NK cells decreases *in vivo* during antiretroviral treatment [9,11,40] and *in vitro* with the addition of IL-2 [12]. Indeed, IL-2 has been shown to help recover perforin and granzyme B expression in NK cells that have killed target cells [39]. Furthermore, one study suggests that IL-2 may be beneficial in restoring CD56⁺CD16⁺ NK cells as they observed an increase in CD56⁺CD16⁺ NK cells and a reduction in CD56^{neg}CD16⁺ NK cells *in vivo* [41]. The *in vivo* efficacy of IL-2 plus antiretroviral therapy requires further exploration. Another interesting approach is the combinatorial use of statins, particularly hydrophilic statins, with cytokine therapies. Statins are known to protect against oxidative stress [42] and hydrophilic statins do not inhibit NK cell cytotoxicity [43]. Such therapies may afford an individual increased control of viral replication prior to initiating antiretroviral therapy and provide increased clearance of viral reservoirs during antiretroviral therapy or in the event of drug resistance. Future studies investigating the effects of cytokine and statin therapies in chronic inflammatory diseases are needed to determine whether they help maintain a healthy NK cell repertoire.

Conclusion

Taken together, CD56^{neg}CD16⁺ NK cells are a mixed population of CD7⁺ NK cells and CD7^{neg} myeloid cells present at a low frequency in healthy donors irrespective of CMV serology status and expanded in HIV-1 viremic subjects. Our results indicate CD7⁺CD56^{neg}CD16⁺ NK cells are activated, mature NK cells that may have recently engaged target cells. Further *in vivo* studies are needed to evaluate treatments for maintaining NK cell functionality in HIV-1 viremic subjects through the use of cytokine and statin therapies.

Methods

Human subjects

Density gradient centrifugation over Ficoll-Paque (GE Healthcare) was used to obtain PBMC from leukocyte concentrates of healthy volunteers (Stanford Blood Center). Additionally, PBMC were obtained from participants in two San Francisco-based HIV-1-infected cohorts, OPTIONS that recruits subjects during early HIV-1 infection [44] and SCOPE that recruits subjects during chronic HIV-1 infection [45]. Table 1 summarizes the subjects used from each HIV-1 cohort and their respective CD4⁺ T cell counts and viral loads. All persons gave informed consent to participate in this study, and the University

of California, San Francisco Committee on Human Research approved this study.

Phenotypic and functional characterization of cell subsets

With the exception of the following antibodies, all fluorophore-labeled antibodies used for phenotypic analysis have been previously described [23]: phycoerythrin (PE)-conjugated anti-Siglec 7 (BioLegend) and PE-conjugated anti-LIR-1 (Beckman Coulter). All stains were performed in the presence of 100 µg/mL human IgG to block Fc receptors. No significant differences in isotype-matched control Ig staining were observed between CD7⁺CD56⁺CD16⁺ NK cells and CD7⁺CD56^{neg}CD16⁺ NK cells. Cells were analyzed by flow cytometry with a four-laser LSR-II instrument (BD Biosciences, San Jose, CA) as previously described [23] and data analyses were carried out using FlowJo flow cytometric analysis software version 9.3.1 (Tree Star, Ashland, OR).

NK cell stimulation and intracellular cytokine staining

NK cell stimulation with K562 target cells or IL-12 plus IL-18, as well as intracellular IFN γ staining, was performed as previously described [23].

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (GraphPad Software). The nonparametric Mann–Whitney *U* test was used to compare between-group distributions with a significance threshold set at *p* < 0.05.

Additional files

Additional file 1: Gating strategy to identify NK cell subsets in a representative healthy donor. Single, live cells were gated on lymphocytes based on forward and side scatter parameters. CD3^{neg}, CD14^{neg}, and CD19^{neg} cells were gated and used to identify classically defined NK cells using CD56 and CD16 expression. To eliminate any potential contaminating myeloid cells, CD7 was assessed on each subset of classically defined NK cells (CD56^{br}CD16^{neg} (green), CD56^{dim}CD16⁺ (red) and CD56^{neg}CD16⁺ (teal)). All three subsets contained CD7^{neg} cells; however, the CD56^{neg}CD16⁺ subset contained the highest proportion of CD7^{neg} non-NK cells. Overlaying the CD7^{neg} non-NK cells onto the CD7⁺ NK cells indicates the high overlap within the subsets and the usefulness of CD7 as an additional marker of NK cells.

Additional file 2: CD7 gating allows precise identification of NK cells. CD56⁺CD16⁺ (red) or CD56^{neg}CD16⁺ (blue) cell subsets were assessed for Nkp30 or granzyme B expression without gating on CD7⁺ cells (left panels) or after gating on CD7⁺ NK cell subsets (right panels).

Additional file 3: Comparison of NK cell gating strategies on Nkp30 and Nkp46 expression. (A) Nkp30 and (B) Nkp46 expression were assessed on NK cells defined in three ways; (1) CD7⁺CD56⁺CD16⁺ NK cells, (2) total CD7⁺CD56⁺ NK cells inclusive of CD56^{bright}CD16^{neg}, CD56^{dim}CD16^{neg} and CD56^{dim}CD16^{pos} NK cells and (3) CD7⁺CD56^{neg}CD16⁺ NK cells.

Abbreviations

NK cell: Natural killer cell; HIV-1: Human immunodeficiency virus-1; DC: Dendritic cell; PBMC: Peripheral blood mononuclear cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JMM designed and performed experiments, analyzed results, made figures, and wrote the manuscript. SL-V. designed and performed experiments and assisted in writing the manuscript. VAY performed experiments and analyzed results. SGD, FMH, and JNM provided reagents and critical revision of the manuscript. LLL and DFN. participated in study design, discussion of the results, and provided critical editing of the manuscript. All authors read and approved the final manuscript.

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